VIROLOGY 216, 202–207 (1996)
ARTICLE NO. 0047

SHORT COMMUNICATION

Beet Soil-Borne Virus RNA 3—A Further Example of the Heterogeneity of the Gene Content of Furovirus Genomes and of Triple Gene Block-Carrying RNAs

R. KOENIG,[1] C. BEIER, U. COMMANDEUR, U. LÜTH, A. KAUFMANN, and P. LÜDDECKE

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Biochemie und Pflanzenvirologie, Messeweg 11, D38104 Braunschweig, Germany

Received August 29, 1995; accepted November 20, 1995

The complete nucleotide sequence of RNA 3 of the Ahlum isolate of beet soil-borne virus (BSBV) was determined from cDNAs obtained with immunocaptured virus particles and denatured preparations of dsRNA. BSBV RNA 3 is unique among the plant virus RNAs studied so far in containing apparently only the coding sequences of a triple gene block (TGB). The derived amino acid sequences of the three putative TGB-encoded proteins showed the highest level of sequence similarities with those of the corresponding proteins of potato mop top furovirus (PMTV) followed by those of peanut clump furovirus and barley stripe mosaic hordeivirus. Progressively fewer similarities were found with the TGB-encoded proteins of beet necrotic yellow vein virus (uncertain classification), potato X potexvirus, and potato M carlavirus. The 3′-terminal 78 nucleotides of BSBV RNA 3 can be folded into a tRNA-like structure and a high degree of sequence similarity exists between the 122 3′-terminal nucleotides of BSBV RNA 3 and PMTV RNA 2. In other regions, however, no pronounced sequence similarities were found between the two RNAs, and PMTV RNA 2 contains an additional putative gene for a cysteine-rich protein downstream of the TGB. The two viruses are unrelated serologically. BSBV RNA 3 adds a further variant to the heterogeneity of the gene content of furovirus genomes and of triple gene block-carrying RNAs.

Beet soil-borne virus (BSBV), which was first isolated from sugar beet in England (*1, 2*) and was later found in many other countries (e.g., *3, 4, 5*), has several properties in common with beet necrotic yellow vein virus (BNYVV), the causal agent of sugar beet rhizomania. A contributory role of BSBV to the rhizomania syndrome has been suggested (*6*). BSBV and BNYVV both have tubular virus particles of several modal lengths, they are transmitted by *Polymyxa betae,* and they have similar host ranges (*7, 2*). However, no relationships have been detected between the two viruses in serological and in cross-hybridization tests and they differ in the number and size of their RNAs (*1, 4, 8, 9*). Also, the RNAs of BNYVV are polyadenylated, whereas those of BSBV (*9*) and of other definitive furoviruses (*10*) have nonpolyadenylated RNAs. Nucleotide sequence analyses have revealed that soil-borne wheat mosaic virus (SBWMV), the type member of the furoviruses, together with other tubular viruses (hordeiviruses, tobraviruses, tobamoviruses), belongs to the tobamovirus lineage of subgroup 3 of positive-strand RNA viruses, whereas BNYVV together with several animal viruses (e.g., rubella virus, hepatitis E virus) forms part of the rubivirus lineage (*11, 12*). BSBV has never been obtained in a highly purified form, but studies with dsRNAs from infected plants suggest that its genome consists of three RNAs. Their sizes vary somewhat between different strains and have been estimated to range between 6.1 and 6.4 kb, 3.0 and 3.6 kb, and 2.6 and 3.3 kb for RNAs 1, 2, and 3, respectively (*8, 9*). Our previous attempts to obtain cDNA clones by using RNA from partially purified preparations of the Ahlum isolate of BSBV as a template had failed. We had been able, however, to clone a number of short cDNA stretches (average size between 200 and 300 nucleotides) when denatured preparations of dsRNA isolated from infected *Chenopodium quinoa* leaves were used as a template (*9*). These clones were now sequenced and served as a basis for determining the complete nucleotide sequence of BSBV RNA 3 from cDNAs obtained from immunocaptured virus particles (*13, 14*) and denatured preparations of dsRNA.

The Ahlum isolate of BSBV (*4*) was propagated on leaves of *C. quinoa* where it causes local lesions only. IC–RT–PCR was done as described (*14*) using reverse transcriptase Superscript II and *Taq* DNA polymerase (both GIBCO BRL) as DNA-synthesizing enzymes. For sequencing, PCR products were purified from agarose

The complete nucleotide sequence of RNA 3 of the Ahlum isolate of beet soil-borne virus (BSBV) has been deposited with the EMBL Data Library under Accession No. Z66493.

[1] To whom reprint requests should be addressed.

0042-6822/96 $12.00

gels using the QIAquick Gel Extraction kit and cloned into the pT7Blue T vector (Novagen). Nucleotide sequences were determined with the Sequenase DNA Sequencing kit, version 2 (United States Biochemical, no. 770770), using α-^{35}S-dATP in the labeling reaction.

The cDNA inserts in the 11 original BSBV RNA 3-specific clones (*9*) were found to form four blocks of partially overlapping nucleotide sequences (Fig. 1, bottom). For each block at least two 20-mer oligonucleotide primers were designed in opposite orientations. Each primer was checked for its ability to initiate cDNA synthesis with immunocaptured virus particles and to yield PCR products in combination with each one of the other primers. PCR products were obtained when primers 21, 12, 5, 7, 18, 9, and 14 (Fig. 1, bottom) were used for cDNA synthesis, suggesting that they were in antisense orientation to the viral RNA. The sequence information gained from PCR products obtained with the primer combinations 12/10, 5/10, 5/11, 5/8$^+$, 7/1, 18/1, and 18/6 allowed us to bridge the gaps between the four blocks. Several primers also initiated cDNA synthesis from heterologous sites to which they were only partially complementary (marked by an asterisk in Fig. 1, bottom). Further sequence information was thus gained with the primer combinations 9*/20, 12*/20, 12*/17, 14*/17, 14*/22, 7*/17, P1*/22, and P1*/17. Primer 14 is complementary to a stretch in BSBV RNA 2 (R. Koenig, unpublished data) and P1 is complementary to nucleotides 2954–2938 of potato mop top virus (PMTV) RNA 2 in a region where considerable sequence similarity is found between RNAs of tymo- and some furoviruses with a tRNA-like structure (*15;* Fig. 2). Further primers were designed in order to encompass regions where sequence readings were difficult. The sequences of the 282 5′-terminal and 29 3′-terminal nucleotides were amplified by means of PCR after poly-dG tailing of the cDNAs to plus-strand RNA from immunocaptured virus particles and to minus-strand RNA from denatured dsRNA preparations using magnetic bead technology (R. Koenig, unpublished). The binding sites of the 3′-biotinylated trapping oligonucleotides used in this approach are marked by triangles in the bottom of Fig. 1. For most parts of BSBV RNA 3 the sequence was read from 3 to 10 separate overlapping cDNA clones. Altogether 46 cDNA clones were sequenced.

Figure 1 (top) shows the complete nucleotide sequence of BSBV RNA 3 (Ahlum isolate) and the amino acid sequences of its putative translation products. For a few positions two different nucleotides were read from different clones. In these cases the nucleotide read from the majority of the clones was incorporated in the sequence shown in Fig. 1 and the other nucleotide was listed on top of it. Some of the differences observed might be due to errors introduced in the sequence by the DNA-synthesizing enzymes or the bacteria, and others may reflect natural variations. The cDNA in one clone lacked one of the five U residues in positions 2478 to 2482 and one of the five A residues in positions 2510 to 2514, suggesting that it originated from a mutant in which a missing U residue had to be compensated for by the elimination of an A residue further downstream apparently in order to allow proper folding of the RNA in this region.

The first nucleotides to be read after the 5′-poly-C stretch (complementary to the poly-dG tail generated on cDNA to BSBV RNA 3) were two G residues. Unfortunately, attempts to generate poly-dC or poly-dA tails on the cDNAs failed, so we cannot completely exclude the possibility that one or a few 5′-C residues are also part of the BSBV RNA 3 sequence. The starting sequence of GGUA$(U)_9$C. . . resembles that in RNAs of many other tubular viruses which, with the exception of BNYVV RNAs (*16, 17, 18*) and tobacco rattle virus RNA 1 (*19*), all start with the sequence GUA. . . (*20, 21, 22, 23, 15, 24, 25, 26, 27*). In the RNAs of the definitive furoviruses the sequence GUA. . . is followed by a varying number of U residues (two to six) which in most cases are followed by a C residue (*23, 24, 25, 26, 27*). In BSBV RNA 3 the U stretch of nine residues is especially long. The following parts of the 404 nucleotide 5′-UTR of BSBV RNA 3 show little sequence similarity with those of other furovirus RNAs.

The 3′-UTR of BSBV RNA 3 extends over a long stretch of 547 nucleotides. It apparently ends with three C residues which were the last to be read before the poly-dG tail generated on the cDNAs to the minus-strand of BSBV RNA 3. The largest possible peptide encoded on this sequence would consist of only 20 amino acids and would have a size of less than 3 kDa. The 78 3′-terminal nucleotides can be folded into a tRNA-like structure which shows considerable sequence similarity to those of RNA 2 of PMTV and to a somewhat lesser extent to those of the RNAs of other furoviruses (Fig. 2), tymoviruses, and sunnhemp mosaic tobamovirus (*28, 15*). The tRNA-like structure of BSBV RNA 3, like those of the other furo- and tymoviral RNAs except for those of PCV, has a possible anticodon for valine. With the PCV RNAs hairpins III (with a possible anticodon sequence for tyrosine) and IV show a deviating structure (Fig. 2). With BSBV RNA 3 and PMTV RNA 2 considerable sequence similarity is also observed in a stretch of ca. 45 nucleotides upstream of the tRNA-like structures, but not in other parts of their 3′-UTRs.

The sequence of BSBV RNA 3 contains three open reading frames (ORFs) starting with nucleotides 405, 1675, and 1889 and ending with nucleotides 1685, 2022, and 2458, respectively. This coding region resembles the triple gene blocks (TGB) found in the genomes of several furo-, hordei-, potex-, and carlaviruses. For white clover mosaic potexvirus (*29*), BNYVV (*30*), and barley stripe mosaic hordeivirus (BSMV) (*31*) the TGB has been shown

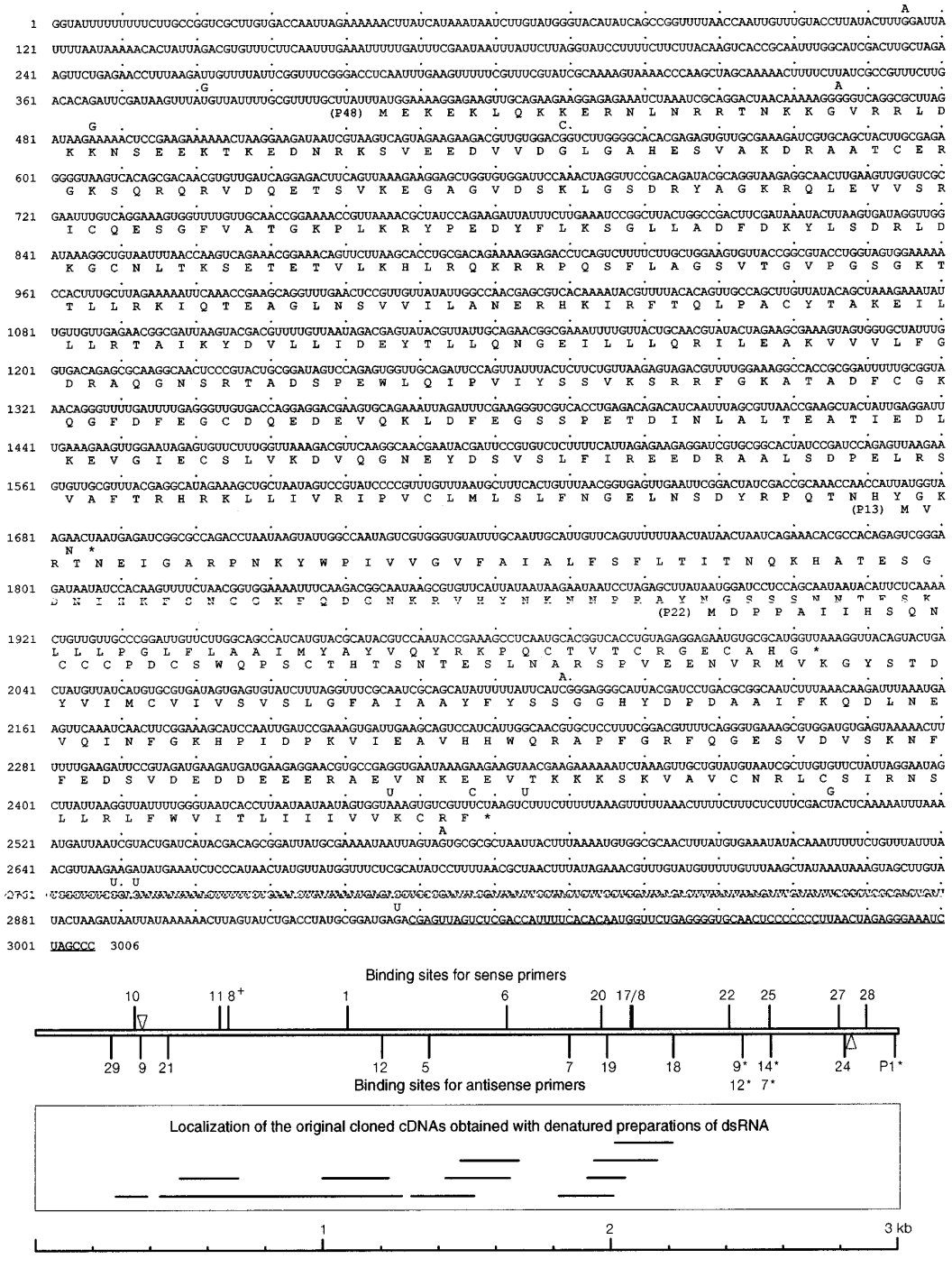

FIG. 1. Top: complete nucleotide sequence of BSBV RNA 3 (Ahlum isolate). In positions where different nucleotides were read from various clones, the nucleotide read most frequently is incorporated in the sequence and the other nucleotide is listed on top of it. The deduced amino acid sequences of the three ORFs are shown below the nucleotide sequence. The 78 3′-terminal nucleotides which can be folded in a tRNA-like structure are underlined. Bottom: Localization of the binding sites for sense and antisense primers and of the original cloned cDNAs obtained with denatured preparations of dsRNA (*9*). Sense primers correspond to nucleotides (in parentheses) 10 (347–366), 11 (644–663), 1 (1088–1107), 6 (1642–1661), 20 (1969–1988), 17 (2070–2089), 8 (2075–2094), 22 (2414–2433), 25 (2554–2575), 27 (2796–2815), and 28 (2893–2917). Primer 8 also binds to the sequence complementary to nucleotides 675–694 (false binding site marked by +). Antisense primers are complementary to nucleotides (in parentheses) 29 (266–244), 9 (368–349), 21 (465–446), 12 (1207–1188), 5 (1371–1352), 7 (1859–1840), 19 (1988–1969), 18 (2218–2199), and 24 (2816–2797). Primers 9, 12, 14, and 7 were also able to initiate cDNA synthesis at nucleotides 2440, 2449, 2540, and 2543, respectively. The false priming sites are indicated by an asterisk. P1 is complementary to nucleotides 2954–2938 of PMTV RNA 2. It initiated cDNA synthesis at nucleotide 2977 of BSBV RNA 3. △ and ▽ mark the binding sites at nucleotides 374–398 (sense) and 2843–2823 (antisense), respectively, for the 3′-biotinylated trapping oligonucleotides used for the amplification of the 5′- and 3′-ends of BSBV RNA 3 (R. Koenig, unpublished).

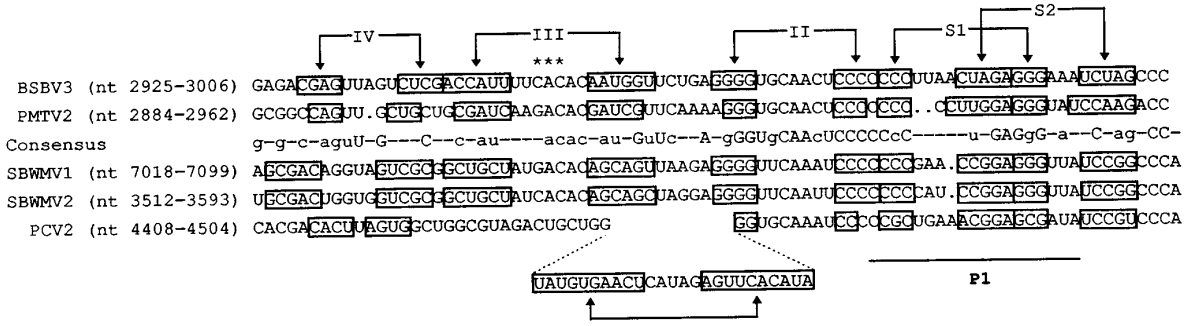

FIG. 2. Comparison of the 3′-terminal sequences of various furovirus RNAs. The consensus sequence for all five RNAs listed is shown below the PMTV RNA 2 sequence in capital letters. Lowercase letters mark nucleotides which, in addition, are identical in BSBV RNA 3 and PMTV RNA 2, but not necessarily in the RNAs of the other viruses. The sequence of PCV RNA 2 (nucleotides 4408–4503) is identical to that of PCV RNA 1 (nucleotides 5802–5897) (*25, 26*). The sequence alignment and the consensus sequence were generated with the programs Pileup and Lineup (Wisconsin Sequence Analysis Package, version 8; *37*). The two stems (S1 and S2) and a hairpin (II) involved in the aminoacyl acceptor arm and two upstream hairpins (III and IV) are indicated for the top four RNAs (*28, 15*). Asterisks mark possible anticodons for valine on the top four RNAs. Hairpins III (with a possible anticodon sequence for tyrosine) and IV of PCV RNAs show a different structure. The binding site of primer P1 on PMTV RNA 2 is indicated. References for nucleotide sequences: PMTV RNA 2 (*15*), PCV RNA 2 (*25*), and SBWMV RNAs 1 and 2 (*27*).

to encode proteins which are involved in virus movement. The amino acid sequences of the putative translation products of the BSBV RNA 3 ORFs are shown in Fig. 3 in comparison with those of the TGB-encoded proteins of other viruses. The dNTP-binding site motif typically occurring in the helicase-like first TGB proteins (*11*) is well conserved in the translation product of the ORF 1 of BSBV RNA 3 (underlined in Fig. 3A). The highest level of sequence similarity of the putative BSBV RNA 3-encoded proteins was found with the corresponding proteins of PMTV, followed by those of PCV (both are furoviruses) and of BSMV, a hordeivirus. The number of identical amino acids became smaller when the TGB-encoded proteins of BNYVV were also included in the comparison and even more so when the corresponding proteins of two filamentous viruses, i.e., potato X potexvirus (PVX) and potato M carlavirus (PVM) were also included. The sequence similarities of the third BSBV TGB-encoded protein with the corresponding proteins of BNYVV, PVX, or PVM were so low that they were not included in Fig. 3C. The first 89 N-terminal amino acids of the first TGB-encoded protein and the last 81 C-terminal amino acids of the third TGB-encoded protein of BSBV show the lowest levels of sequence similarity with the corresponding proteins of other viruses.

From Fig. 4 it is evident that the size of the second TGB-encoded protein (12 to 14 kDa) is rather similar for furo-, hordei-, and other tubular as well as for filamentous viruses. The first and the third TGB-encoded proteins, however, are much larger with furo- and other tubular viruses than with the filamentous PVX and PVM. Figure 4 also illustrates the variability of the gene content of TGB-carrying RNAs. BSBV RNA 3 is unique in apparently containing no further genes besides the TGB. PMTV RNA 2 contains in addition to the TGB a putative gene for a cysteine-rich protein (*24*). Coding regions for functionally possibly similar cysteine-rich proteins are also found on the 3′-ends of the TGB-carrying RNAs of BNYVV (*17, 32*) and PVM (*33*). The viral coat protein gene is found upstream of the TGB in PCV RNA 2 (*25*), BSMV RNA β (*20*), and BNYVV RNA 2 (*17*) and downstream of it in the RNAs of PVX (*34*) and PVM (*33*). A readthrough protein which carries the coat protein on its N-terminus and is involved in the initiation of encapsidation (*35*) as well as in *Polymyxa* transmission (*36*) and a separate 39-kDa protein with unknown functions (*25*) are also encoded upstream of the TGB in BNYVV RNA 2 and PCV RNA 2, respectively. In the monopartite genomes of the filamentous PVX (*34*) and PVM (*33*) the TGB is preceded on the 5′-end by a large ORF, presumably encoding enzymes involved in the replication of the viral RNA.

BSBV RNA 3 adds another variant to the organizational heterogeneity of the genomes of viruses which have been considered to be definitive or possible furoviruses (*10*). As shown in Fig. 4 the definitive furoviruses PMTV and PCV and the possible furovirus BNYVV all differ in the gene content of their TGB-carrying RNAs. SBWMV, the type member of the furoviruses, lacks a TGB altogether. It seems to be replaced by a single gene at the 3′-end of its RNA 1 which encodes a 37-kDa protein resembling the cell to cell transport proteins of dianthoviruses (*27*).

In the deduced amino acid sequences of its three putative TGB-encoded proteins and the sequence of its 122 3′-terminal nucleotides BSBV RNA 3 shows the closest relationships to PMTV RNA 2. In other regions, however, no strong sequence similarities were found between the two RNAs (results not shown) and PMTV RNA 2 contains in addition to the TGB a putative gene for a cysteine-rich protein (*24*). It will be interesting to see whether close relationships between BSBV and PMTV can also be found with their other RNAs. At least for the coding region

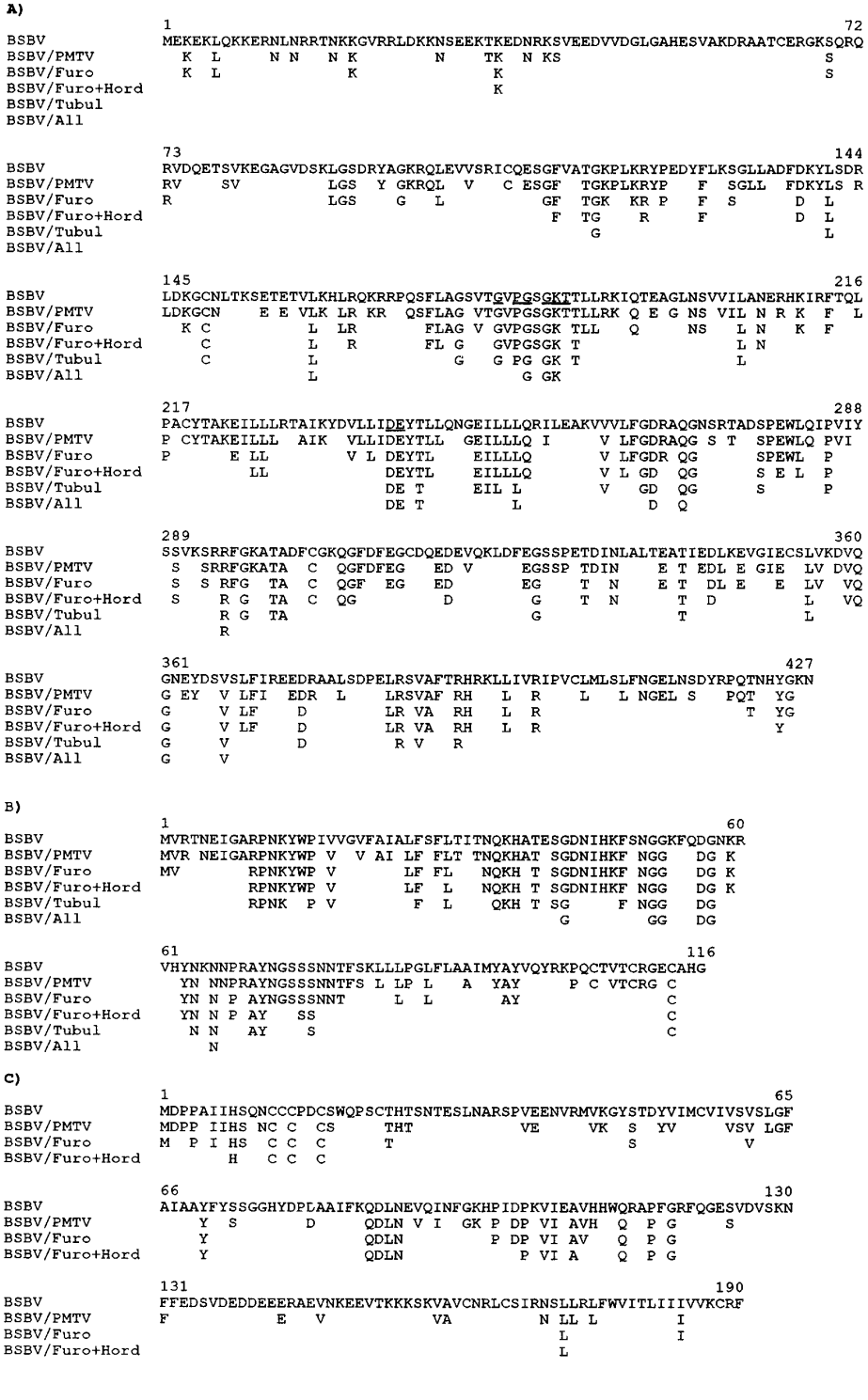

FIG. 3. (A, B, and C) Comparison of the deduced amino acid sequences of the putative proteins encoded on ORFs 1, 2, and 3, respectively, of BSBV RNA 3 with those of the respective proteins of other viruses. The consensus sequences and alignments were generated with the programs Pileup and Lineup (Wisconsin Sequence Analysis Package, version 8; *37*). Meanings of designations: BSBV/PMTV, amino acids identical in BSBV and PMTV proteins; BSBV/Furo, amino acids identical in BSBV, PMTV, and PCV proteins; BSBV/Furo+Hord, amino acids identical in BSBV, PMTV, PCV, and BSMV proteins; BSBV/Tubul, amino acids identical in BSBV, PMTV, PCV, BSMV, and BNYVV proteins; BSBV/all, amino acids identical in BSBV, PMTV, PCV, BSMV, BNYVV, PVX, and PVM proteins. In (C) the lines "BSBV/Tubul" and "BSBV/all" have been omitted because of the almost complete lack of identical amino acids. The dNTP-binding motif on the putative translation product of ORF 1 of BSBV RNA 3 is underlined. References for nucleotide sequences: PMTV RNA 2 (*24*), PCV RNA 2 (*25*), BSMV RNA β (*20*), BNYVV RNA 2 (*17*), PVX RNA (*34*), and PVM RNA (*33*).

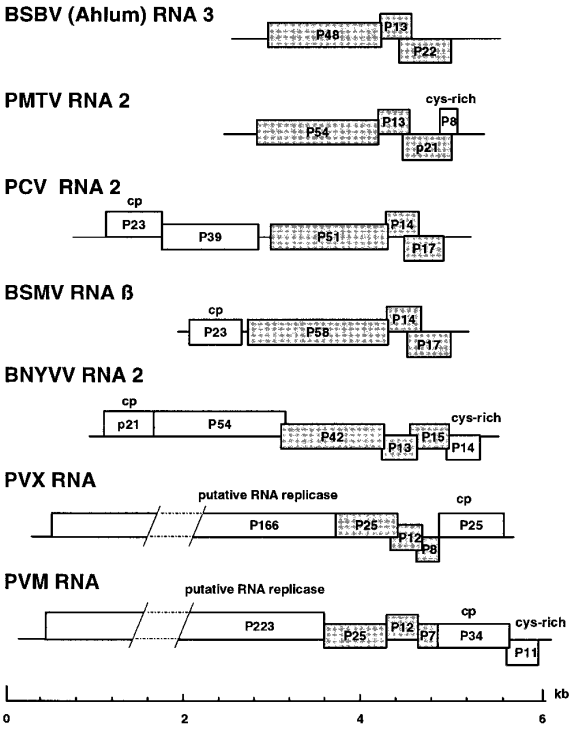

FIG. 4. Comparison of the gene content of the TGB-containing RNAs of various plant viruses. TGB ORFs are highlighted by a gray underlay. References for nucleotide sequences are as in Fig. 3.

of the coat protein this is unlikely, because the two viruses are unrelated serologically (*1*).

BSBV RNA 3 may be the first example of a plant viral RNA for which the nucleotide sequence has been determined entirely from cDNAs obtained with immunocaptured virus particles and with dsRNA, which can both be readily obtained from crude plant sap. This approach may also be useful for other viruses which are purified only with difficulty. The BSBV antiserum we have used also showed some reactivity with normal plant constitutents, indicating that the immunocapture technique does not require antisera to highly purified virus.

ACKNOWLEDGMENT

The financial support given by the German Ministry of Research and Technology (Grant 0310582A) is gratefully acknowledged.

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